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Title of Invention	Method for Genotyping Single Nucleotide Polymorphisms

Application Number :

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Documents being submitted:	Files
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	us-request.dtd
	us-request.xsl
us-fee-sheet	SNPSPP-usfees.xml
	us-fee-sheet.xsl
	us-fee-sheet.dtd
application-body	SNPSPP-trans.xml
	us-application-body.xsl
	application-body.dtd
	wipo.ent
	mathml2.dtd
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Description

Method for Genotyping Single Nucleotide Polymorphisms

BACKGROUND OF INVENTION

[0001] DNA analysis is becoming increasingly important in the diagnosis of hereditary diseases, detection of infectious agents, tissue typing for histocompatability, identification of individuals in forensic and paternity testing, and monitoring the genetic makeup of plants and animals in agricultural research (Alford, R. L., et al., Curr Opn. Biotechnol (1994) 5:29-33). In addition, DNA analysis is crucial in large-scale genetic studies to identify susceptibility alleles associated with common diseases involving multiple genetic and environmental factors (Risch, N., et al., Science (1996) 273:1516-1517). Recently, attention is focused on single nucleotide polymorphisms (SNPs), the most common DNA sequence variation found in mammalian genomes (Cooper, D. N., et al., Hum Genet (1985) 69:201-205). While most of the SNPs do not give rise to

detectable phenotypes, a significant fraction of them are disease-causing mutations responsible for genetic diseases. As the DNA sequence of the human genome is completely elucidated, large-scale DNA analysis will play a crucial role in determining the relationship between genotype (DNA sequence) and phenotype (disease and health) (Cooper, D. N., et al., Hum Genet (1988) 78:299-312). Although some assays have considerable promise for high throughput, the recently developed DNA diagnostic methods, including the high-density chip arrays for allele-specific hybridization analysis (Pease, A. C., et al., Proc Natl Acad Sci USA (1994) 91:5022-5026; Yershov, G., et al., Proc Natl Acad Sci USA (1996) 93:4913-4918), Wang, D. G., et al., Science (1998) 280:1077-1081, the homogeneous 5'-nuclease allele-specific oligonucleotide cleavage assay (TaqMan ASO, Livak, K. J., et al., Nat Genet (1995) 9:341-342), Whitcombe, D., et al., Clin Chem (1998) 44:918-923 a homogeneous fluorescence assay for PCR amplifications: its application to real-time, single-tube genotyping, the homogeneous template-directed dye-terminator incorporation (TDI) assay (Chen, X., et al., Nucleic Acids Res (1997) 25:347-353; Chen, X., et al., Proc Natl Acad Sci USA (1997) 94:10756-10761) the homoge-

neous dye-labeled oligonucleotide ligation (DOL) assay (Chen, X. et al. Genome Research (1998) 8: 549-556.), and the homogeneous molecular beacon ASO assay (Tyagi, S. et al. Nature Biotechnology (1998) 16: 49-53), all require specialty reagents and expensive detection instrumentation. All the DNA diagnostic methods listed above involve amplification of target sequences to increase the sensitivity and specificity of the assays through polymerase chain reaction (PCR) or other similar amplification technologies. For example, one of the best-known amplification methods is the polymerase chain reaction (referred to as PCR) which is described in detail in U.S. Pat. Nos. 4,683,195, 4,683,202 and 4,800,159. Briefly, in PCR, two primer sequences are prepared which are complementary to regions on opposite complementary strands of the target sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a thermostable DNA polymerase (e.g., Taq polymerase). If the target sequence is present in a sample, the primers will bind to the target and the polymerase will cause the primers to be extended along the target sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers

reaction products will dissociate from the target to become new targets. The excess primers will bind to the target and to the reaction products and the process is repeated. Other technologies result in amplification of a target sequence by strand displacement. These techniques include an enzymatic 'nicking' or preferential cleavage of one of two strands present in a double-stranded DNA recognition site and the separation or detection of amplified products that include the target site, which is presented in U.S. Pat. Nos. 5,270,184 and 5,455,166 and each of which is hereby incorporated by reference herein. Still other techniques employ the use of a fluorescently labeled primer and detect fluorescence or fluorescence polarization after the primer is hybridized to the target region, which is presented in U.S. Pat. No. 5,593,867. In U.S. Pat. No. 5,641,633, double-stranded DNA binding protein is also used to further preserve the hybridization of the fluorescently labeled primer to the target site. These methods require the use of fluorescently labeled primers and their detection while hybridized to the target site. Template-directed primer extension is a chain terminating DNA sequencing protocol designed to ascertain the nature of the one base immediately 3' to the sequencing primer

that is annealed to the target DNA immediately upstream from the polymorphic site. In the presence of DNA polymerase and the appropriate terminator, e.g. a dideoxyribonucleoside triphosphate (ddNTP), the primer is extended specifically by one base as dictated by the target DNA sequence at the polymorphic site. By determining which ddNTP is incorporated, the allele(s) present in the target DNA can be inferred. This genotyping method has been widely used in many different formats and proven to be highly sensitive and specific (Syvanen, A.-C et al, Genomics (1990) 8: 684-692; Syvanen, A.-C. and Landegren, U. Human Mutation (1994) 3: 172-179).

[0002] US patents US 6,180,408 and 6,440,707describe a new method for DNA diagnostics based on template-directed primer extension and detection by fluorescence polarization (TDI-FP). In this method, amplified genomic DNA fragments containing polymorphic sites are incubated with a oligonucleotide primer designed to hybridize to the DNA template adjacent to the polymorphic site in the presence of allelic dye-labeled terminator and a modified DNA polymerase. The primer is extended by the dye-terminator specific for the allele present on the template. At the end of the reaction, the fluorescence polarization of

the two dye-terminators in the reaction mixture is analyzed directly without separation or purification. This homogeneous DNA diagnostic method is shown to be highly sensitive and specific and is suitable for automated genotyping of large number or samples.

[0003] It has been observed with the TDI-FP assay that in some cases the assay is not as robust as in other cases requiring modification to the recommended reaction process, generally this involves performing fewer or more cycles during the TDI process. What has been observed is that some of the genotypic clusters do not produce a stable signal and migrate to a new X-Y coordinates (when data presented as a graph of the polarity (units of mP) of Dye 1 vs. Dye 2) with increasing cycle producing erroneous outcomes. Unlike the PCR primers, which have some flexibility in their design, SNP primers are extremely limited, especially on the 3'-end that must end either one base removed from or cover the SNP site of interest. Increasing reaction robustness must occur by some other mechanism than redesign of the primer. In other examples, additional steps or reagents have been suggest to increase assay performance such as changing the fluorescent dyes, adding methanol, or even additional proteins such as sin-

gle-stranded DNA binding protein (SSB). In even more drastic cases, changing the mode of detection from FP to another measurement modes has been proposed, e.g. fluorescence quencing.

[0004] As described in Tabor/Richardson patent US5,498,523 DNA sequencing is a primer extension reaction in which an oligonucleotide primer having homology to a single-stranded template DNA, e.g., genomic DNA, is caused to anneal to the template DNA. The annealed mixture is then provided with a DNA polymerase in the presence of 2'-deoxynucleoside triphosphates (dNTPs) under conditions in which the DNA polymerase extends the primer to form a complementary DNA strand to the template DNA. In a DNA sequencing reaction, the primer is extended additionally in the presence of a chain-terminating agent, e.g., a dideoxynucleoside triphosphate, to cause base-specific termination of the primer extension. Sanger et al., 74 Proc. Nat'l. Acad. Sci. 5463, 1977. In a polymerase chain reaction two primers are provided, each having homology to opposite strands of a double-stranded DNA molecule, and after the primers are extended via the polymerase and dNTPs, they are separated from their templates, and additional primers caused to anneal to the

templates and the extended primers. The additional primers are then extended via the polymerase and dNTPs. The steps of separating, annealing, and extending are repeated in order to amplify the number of copies of template DNA. Saiki et al., 239 Science 487, 1988. In both of these types of reactions that utilize 2'-deoxynucleoside triphosphates, a molecule of inorganic pyrophosphate (PPi) is released per addition of the dNTP via the DNA polymerase. Thus in any given reaction 10 1000's of molecules of PPi are released per extension reaction from a given primer.

[0005] Also described in Tabor/Richardson, US 5,498,523, pyrophosphorolysis is the process where a polymerase performs a reverse polymerization reaction. One condition which is permissive for the pyrophosphorolysis process is that the 3'-terminal base of the oligonucleotide primer match via the Watson-Crick base pairing rules (A:T, G:C) to the template nucleic acid. This condition is also critical to most reactions employing SNP analysis by the method(s) of SBE. The pyrophosphorolysis is caused and only permitted by the availability of pyrophosphate. For example, PCR is inhibited by addition of pyrophosphate even at very low concentrations. Providing an agent, for

example, a pyrophosphatase, capable of removing pyrophosphate, can prevent this pyrophosphorolysis. Addition of pyrophosphatase to a PCR greatly enhances the progress of that reaction, and provides superior results compared to use of the method without a pyrophosphatase. Similarly addition of a pyrophosphatase to a DNA sequencing reaction provides more uniformity in intensities of bands formed in a polyacrylamide gel used to identify products of the sequencing reaction. This uniformity is due to prevention of degradation of specific DNA products by pyrophosphorolysis.

[0006] A method described in US6,534,269 (Liu and Sommers) actually exploits the mechanism of pyrophosphorolysis for SNP analysis and as explained is a novel method which couples reverse:forward reactions of the polymerase to give a process called pyrophosphorolysis activated polymerization (PAP). In PAP, pyrophosphorolysis and polymerization by DNA polymerase are coupled serially for each amplification by using an activatable oligonucleotide P* that has a non-extendable 3'-deoxynucleotide at its 3' terminus. PAP can be applied for exponential amplification or for linear amplification. PAP can be applied to amplification of a rare allele in admixture with one or more wild

type alleles by using an activatable oligonucleotide P* that is an exact match at its 3' end for the rare allele but has a mismatch at or near its 3' terminus for the wild type allele. PAP is inhibited by a mismatch in the 3' specific subsequence as far as 16 nucleotides away from the 3' terminus. PAP can greatly increase the specificity of detection of an extremely rare mutant allele in the presence of the wild type allele. Specificity results from both pyrophosphorolysis and polymerization since significant nonspecific amplification requires the combination of mismatch pyrophosphorolysis and misincorporation by the DNA polymerase, an extremely rare event. Using genetically engineered DNA polymerases greatly improves the efficiency of PAP.

[0007] All publications cited are incorporated in their entirety by reference herein.

DETAILED DESCRIPTION

[0008] I have determined that the modification of removing inorganic pyrophosphate from a single base primer extension reaction in the strategy for the TDI assay allows for the rapid analysis of DNA sequence variations, including SNPs and unique insertions/deletions, in a homogeneous assay using a very inexpensive enzyme, inorganic pyrophos-

phatase, which eliminates the need for specialty reagents or expensive instruments. This present approach improves the specificity of enzymatic discrimination between the two alleles of a DNA sequence variation in a template-directed primer extension reaction with a higher degree of precision. This invention is applicable to methods which are all variations of a single base primer extension reaction (SBE), defined only as requiring minimally a polymerase, a nucleic acid template (pre- or post amplification), an oligonucleotide primer directing to the target site of analysis (e.g., SNP, addition, deletion or other genetic aberration), nucleoside triphosphate analogs (e.g., ddNTPs, acyNTPs, or other 3'-deoxynucleoside analogs) with or without a detectable labels attached, are all improved by the method of the invention which removes inorganic pyrophosphate from the SBE reaction. The SBE reaction may be performed in single tubes, multiwell microplates, microchannel devices, flat surfaces with or without depressions, or upon microarrays. The method of this invention most importantly improves those assays which have been developed to minimize physical transfer steps and or washing steps and are most commonly referred to as a homogeneous reaction (all step(s) per-

formed sequentially in a single reaction vessel).

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Claims

[c1] A method for genotyping single nucleotide polymorphisms using the process of single base extension, the improvement comprising the use of an enzyme that degrades inorganic pyrophosphate.

[c2] The enzyme of claim 1 in which the enzyme is inorganic pyrophosphatase (IUBMB Enzyme Nomenclature EC 3.6.1.1, Common name: inorganic diphosphatase, Reaction: diphosphate + H_2O = 2 phosphate)

[c3] The method of claim 1 in which the degradation of inorganic pyrophosphate by the enzyme inorganic pyrophosphatase is done as a separate unique step of the analysis process.

[c4] The method of claim 1 in which the degradation of inorganic pyrophosphate by the enzyme inorganic pyrophosphatase is combined with one or more steps of the analysis process.

[c5] The method of claim 1 where the single base extension reaction utilizes a DNA polymerase, a primer that has a 3'-end immediately upstream (5') of the interrogation site, nucleoside triphosphate terminators or analogs

which act as terminators, and DNA from biological sample either before or after amplification.

[c6] The method of claim 1 where the single base extension reaction utilizes a thermostable DNA polymerase, a primer that has a 3'-end immediately upstream (5') of the interrogation site, nucleoside triphosphate terminators or analogs which act as terminators, and DNA from biological sample either before or after amplification.

[c7] The method of claim 1 where the single base extension reaction utilizes a mutant, thermostable DNA polymerase, a primer that has a 3'-end immediately upstream (5') of the interrogation site, nucleoside triphosphate terminators or analogs which act as terminators, and DNA from biological sample either before or after amplification.

[c8] The method of claim 1 where the single base extension reaction utilizes a mutant, thermostable DNA polymerase, a primer that has a 3'-end immediately upstream (5') of the interrogation site, 2', 3'-dideoxynucleoside triphosphate terminators and DNA from biological sample either before or after amplification.

[c9] The method of claim 1 where the single base extension

reaction utilizes a mutant, thermostable DNA polymerase, a primer that has a 3'-end immediately upstream (5') of the interrogation site, acycloterminators and DNA from biological sample either before or after amplification.

[c10] The method of nucleic acid amplification in any of the above claims, 4-8, which utilize a polymerase and nucleoside triphosphates.

[c11] The method of claim 10 utilizing a DNA polymerase and 2'deoxynucleotide triphosphates.

[c12] The method of claim 10 utilizing a RNA polymerase and ribonucleotide triphosphates.

[c13] The method of claim 10 where the DNA amplification is by the method of polymerase chain reaction (PCR).

[c14] The method of claims 7-9 in which the mutant DNA polymerase is from either a Family A (e.g. *E coli* pol 1, T7 or *TAQ*) or Family B (e.g. hyperthermophilic archaeon VENT, Deep Vent, 9°N, *Pfu*) DNA polymerase.

[c15] The method of claim 1 in which the single base extension reaction utilizes terminators that are chemically labeled in way that allows their detection.

[c16] The method of claim 15 where the label(s) is radioactive.

[c17] The method of claim 15 where the label is fluorescent and method of detection is by any of the following: direct fluorescence, fluorescence quenching, fluorescence polarization, fluorescence anisotropy, time resolve fluorescence or fluorescence energy transfer.

[c18] The method of claim 17 where there are from 1 to 4 different fluorophores.

[c19] The method of claim 15 where the label is a mass tag used in conjunction with mass spectrometry.

[c20] The method of claim 15 where the label is an antigen, hapten, or ligand for an antibody or binding partner (receptor).

[c21] The method of claim 20 where there are from 1 to 4 different antigen, hapten, ligand labels.

[c22] The method of claim 15 where the label is an enzyme.

[c23] The method of claim 22 where there are from 1 to 4 different enzymes.

[c24] The method of claim 1 in which the single base extension reaction utilizes terminator bases (adenine, deazaadenine, deazaguanine, cytosine, thymidine/uridine), which are not carrying bulky, chemical reporter groups

but are detected by means of atomic mass or electrophoretic mobility.

[c25] The method of claim 1 where the pyrophosphatase degradation of inorganic pyrophosphate produced in some previous reaction step is done prior to the step of the single base extension reaction.

[c26] The method of claim 25 where the pyrophosphatase degradation is performed concurrent with a nucleic acid amplification reaction that produces inorganic pyrophosphate.

[c27] The method of claim 25 where the pyrophosphatase degradation is performed following the nucleic acid amplification and prior to the single base extension reaction.

[c28] The method of claim 1 where the pyrophosphatase degradation of inorganic pyrophosphate is concurrent with the step of the single base extension reaction.

[c29] The method of claim 1 where the pyrophosphatase is a non-thermostable version of the enzyme.

[c30] The method of claim 1 where the pyrophosphatase is a thermostable version of the enzyme.

[c31] The method of claim 1 wherein the genotyping process

involves no physical separation of starting reactants or reaction products between analysis steps.

[c32] The method of claim 31 where all of the reaction steps are performed sequentially in a single tube, multi-well plate or slide, or on a flat surface containing an array of samples to be analyzed.

[c33] The method of claim 1 where the single base extension in which a biological sample containing one or more SNPs of interest is analyzed is by the method of TDI-FP (Template-directed Dye-terminator Incorporation with Fluorescence Polarization detection).

[c34] The method of claim 33 in which the inorganic pyrophosphatase is adding during the PCR.

[c35] The method of claim 33 in which the inorganic pyrophosphatase is adding during the PCR clean up stage employing the use of the enzymes: exonuclease and shrimp alkaline phosphatase.

[c36] The method of claim 33 in which the inorganic pyrophosphatase is adding during the TDI-FP step.

[c37] The method of claims 34 or 36 in which the inorganic pyrophosphatase is a thermostable DNA polymerase.

[c38] The method of claim 33 in which the terminators are 2',

3'-dideoxynucleotides.

[c39] The method of claim 33 in which the terminators are acycloterminators.

[c40] The method of claim 1 where the single base extension in which a biological sample containing one or more SNPs of interest is analyzed is by the method of APEX (Arrayed Primer Extension).

[c41] The method of claim 40 in which the inorganic pyrophosphatase is adding during the PCR.

[c42] The method of claim 40 in which the inorganic pyrophosphatase is adding during the PCR clean up stage employing the use of the enzymes: exonuclease and alkaline phosphatase.

[c43] The method of claim 40 in which the inorganic pyrophosphatase is adding during the APEX step.

[c44] The method of claims 41 or 43 in which the inorganic pyrophosphatase is a thermostable DNA polymerase.

[c45] The method of claim 41 in which the terminators are 2', 3'-dideoxynucleotides.

[c46] The method of claim 41 in which the terminators are acycloterminators.

[c47] The method of claim 1 where the single base extension reaction utilizes a DNA polymerase, a primer that has a 3'-end which hybridizes with (covers) the SNP interrogation site, nucleoside triphosphate terminators or analogs which act as terminators, and DNA from biological sample either before or after amplification and the usual method of analysis is determined by whether or not the primer is extended one based depending upon whether or not the primer was 100% hybridized.

[c48] The method of claim 47 where the terminator is labeled by a fluorophore and incorporation is measure by any of the following: direct fluorescence, fluorescence quenching, fluorescence polarization, fluorescence anisotropy, time resolve fluorescence or fluorescence energy transfer.

[c49] A kit for performing the process of genotyping by the method of single base extension which includes: exonuclease, shrimp alkaline phosphatase or other heat labile equivalent, inorganic pyrophosphatase, a DNA polymerase, nucleotide terminators (modified or not) or other terminator equivalents, buffers and protocol.

[c50] A kit as per claim 49, which additionally includes the reagents, enzymes, buffers and instructions for perform-

ing the nucleic acid amplification process.

[c51] A kit as per claim 49, which includes as a single tube a mixture of the exonuclease, alkaline phosphatase, and inorganic pyrophosphatase in a suitable buffer.

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Method for Genotyping Single Nucleotide Polymorphisms

Abstract

This invention relates to improved methods for diagnostic procedures and diagnostic kits for the analysis of DNA variations, especially single nucleotide polymorphisms (SNPs), employing a procedure to enzymatically remove inorganic pyrophosphate from the sample prior to or during one of many variations of single base extension (SBE) reactions using a polymerase and chemical moieties which function as nucleoside triphosphate terminators. This improvement is especially useful in template-directed primer extension reactions that have been developed which do not utilize any intermediate separation or transfer steps between stages of the analysis. Also, this invent

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